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## Unanchored K48-linked poly-ubiquitin synthesized by the E3-ubiquitin ligase TRIM6 stimulates the interferon- $\text{IKK}\epsilon$ kinase mediated antiviral response

Ricardo Rajsbaum<sup>1,2,\*</sup>, Gijs A. Versteeg<sup>1,2,4,\*</sup>, Sonja Schmid<sup>1</sup>, Ana M. Maestre<sup>1</sup>, Alan Belicha-Villanueva<sup>1,2</sup>, Carles Martínez-Romero<sup>1,2</sup>, Jenish R. Patel<sup>1,2</sup>, Juliet Morrison<sup>1,2</sup>, Giuseppe Pisanelli<sup>1,2,5</sup>, Lisa Miorin<sup>1,2</sup>, Maudry Laurent-Rolle<sup>1,2</sup>, Hong M. Moulton<sup>6</sup>, David A. Stein<sup>6</sup>, Ana Fernandez-Sesma<sup>1,2</sup>, Benjamin R. tenOever<sup>1,2</sup>, and Adolfo García-Sastre<sup>1,2,3,†</sup>

<sup>1</sup>Department of Microbiology, Icahn School of Medicine at Mount Sinai, One Gustave L. Levy Place, New York, NY 10029, USA

<sup>2</sup>Global Health and Emerging Pathogens Institute, Icahn School of Medicine at Mount Sinai, One Gustave L. Levy Place, New York, NY 10029, USA

<sup>3</sup>Department of Medicine, Icahn School of Medicine at Mount Sinai, One Gustave L. Levy Place, New York, NY 10029, USA

<sup>4</sup>Max F. Perutz Laboratories, University of Vienna, Dr. Bohr-Gasse 9/4, 1030, Vienna, Austria

<sup>5</sup>Department of Veterinary Medicine and Animal Production, University of Naples Federico II, Via Federico Delpino 1, 80137, Naples, Italy

<sup>6</sup>Department of Biomedical Sciences, College of Veterinary Medicine, Oregon State University, Corvallis, OR 97331

### Summary

Type-I interferons (IFN-I) are essential antiviral cytokines produced upon microbial infection. IFN-I elicits this activity through the upregulation of hundreds of IFN-I stimulated genes (ISGs). The full breadth of ISG induction demands activation of a number of cellular factors including the  $\text{I}\kappa\text{B}$  kinase epsilon ( $\text{IKK}\epsilon$ ). However, the mechanism of  $\text{IKK}\epsilon$  activation upon IFN receptor signaling has remained elusive. Here we show that TRIM6, a member of the E3-ubiquitin ligase tripartite motif (TRIM) family of proteins, interacts with  $\text{IKK}\epsilon$  and promotes induction of  $\text{IKK}\epsilon$ -

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<sup>†</sup>Corresponding author. Mailing address: Department of Microbiology, Icahn School of Medicine at Mount Sinai, One Gustave L. Levy Place, New York, NY 10029. Phone: (212) 241-7769. Fax: (212) 534-1684. adolfo.garcia-sastre@mssm.edu.

<sup>\*</sup>These authors contributed equally to this work.

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dependent ISGs. TRIM6 and the E2-ubiquitin conjugase Ube2K cooperate in the synthesis of unanchored K48-linked poly-ubiquitin chains, which activate IKK $\epsilon$  for subsequent STAT1 phosphorylation. Our work attributes a previously unrecognized activating role of K48-linked unanchored poly-ubiquitin chains in kinase activation and identifies the Ube2K-TRIM6-ubiquitin axis as critical for IFN signaling and antiviral response.

## Introduction

Innate immune signaling pathways are activated when pathogen associated molecular patterns (PAMPs) contained in microbial products are recognized by host cell pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) and RIG-I-like receptors (RLRs) (Medzhitov et al., 1997; Meylan et al., 2006). Activation of these pathways ultimately results in production of type I interferons (IFN-I) and other cytokines essential for an effective anti-microbial response (Akira et al., 2006). Different adaptor proteins function to transmit downstream signals converging at the point of the I $\kappa$ B (IKK) and IKK-related kinases (Kawai et al., 2005; Yamamoto et al., 2003). The IKK-related kinases (TBK1 and IKK $\epsilon$ ) phosphorylate the transcription factors IRF3 and IRF7 required for type I IFN production (Hemmi et al., 2004; Sharma et al., 2003).

IFN-I receptor-mediated signaling triggers phosphorylation of signal transducers and activators of transcription 1 (STAT1) and STAT2. Together STAT1, STAT2, and a third transcription factor, IRF9, form the Interferon Stimulated Gene Factor 3 (ISGF3) complex, which is essential for induction of many IFN stimulated genes (ISGs) (Platanias, 2005). The tyrosine kinases JAK1 and TYK2 are critical for phosphorylation of tyrosine 701 (Y701) on STAT1 and its subsequent induction of a broad range of ISGs (Shuai et al., 1993). In addition to tyrosine phosphorylation, S708 has also been reported to modulate the transcriptional potential of STAT1 and is required for establishing an effective antiviral state (Tenover et al., 2007). S708 is phosphorylated by IKK $\epsilon$  during IFN signaling, but the mechanism by how IKK $\epsilon$  becomes activated has remained elusive (Tenover et al., 2007).

Ubiquitination of proteins is an important post-translational covalent modification process which has been thoroughly demonstrated to regulate signaling pathways in immune regulation and cytokine production (Jiang and Chen, 2012). Ubiquitin (Ub), a 76 amino acid protein, has seven lysines, each of which can be conjugated by another Ub to form a poly-Ub chain (Trempe, 2011). It is generally accepted that proteins covalently attached to poly-Ub chains linked through lysine 48 (K48) of Ub are targeted for degradation by the proteasome. Conversely, protein modification with K63-linked poly-Ub chains has non-proteolytic activating functions (Chen and Sun, 2009).

The TRIM family of proteins, which are characterized by the presence of a RING, B box and a coiled-coil domain, has been implicated in innate immune signaling pathways by acting as E3-Ub ligases (Rajsbaum et al., 2014). Recently, we have shown that an unprecedented large number of TRIMs positively regulates innate immune responses (Versteeg et al., 2013), however, only a limited number of these TRIMs that act as positive regulators have been characterized in detail. In this study we report that TRIM6 together with the E2 Ub conjugase Ube2K synthesizes unanchored K48-linked poly-Ub chains which

activate IKK $\epsilon$  to phosphorylate STAT1 S708, ultimately resulting in the induction of a subset of ISGs essential for the antiviral response *in vitro* and *in vivo*.

## Results

### TRIM6 plays a role in induction of IFN $\beta$ and ISGs but not of NF- $\kappa$ B dependent cytokines

We and others have recently reported that a large number of TRIMs are able to positively enhance innate immune signaling pathways (Versteeg et al., 2013; Uchil et al., 2013). TRIM6, which had thus far remained completely uncharacterized in immune regulation, was identified as a positive regulator of the type I IFN axis in our screen (Versteeg et al., 2013). We thus set out to investigate the role of TRIM6 in the activation of these innate immune pathways.

Firstly, the ability of TRIM6 to enhance RIG-I-mediated activation of IFN and NF- $\kappa$ B responsive promoters was investigated (Figure 1A, 1B and S1A). Exogenous TRIM6 expression enhanced the Sendai virus (SeV)- and the constitutively active RIG-I(2CARD)-dependent IFN response in a dose-dependent manner (Figure 1A). In contrast, RIG-I(2CARD)-dependent induction of the NF- $\kappa$ B promoter was minimally affected as compared to TRIM25 which has been previously reported to enhance RIG-I-dependent NF- $\kappa$ B activation (Gack et al., 2007) (Figure 1A). These results suggest that TRIM6 is a positive regulator of the IFN $\beta$  pathway acting downstream of MAVS/IPS-1 towards the TBK-1 and IKK $\epsilon$  signaling axis (Figure S1A). Accordingly, TRIM6 also enhanced TBK-1- and IKK $\epsilon$ -dependent IFN induction (Figure 1B).

To further validate these results, we performed loss of function experiments by silencing TRIM6 expression with short-hairpin lentiviral vector pools (shRNA) in primary human monocyte-derived dendritic cells (hDCs), from four different healthy human donors (Figure S1B). TRIM6 mRNA expression was consistently reduced over 80% in TRIM6-silenced (TRIM6<sup>si</sup>) hDCs (Figures 1C and S1C). At early time points after TLR4 activation by LPS (2 h p.t), IFN $\beta$ , ISG54, TNF $\alpha$ , IL-6 and IL-8 mRNA levels were induced about 10-80 fold in control cells compared to mock-treated samples (Figures 1D and S1D). Strikingly, while IFN $\beta$  and ISG54 mRNA levels were attenuated in TRIM6<sup>si</sup> hDCs as compared to control cells, IL-6 and IL-8 levels were not affected and in some cases even increased (Figures 1E and S1E). These data indicate that TRIM6 specifically acts as a positive regulator of the IFN system, yet does not play a role in promoting induction of pro-inflammatory cytokines.

Next, we set out to establish the importance of TRIM6 for mounting a cytokine response during virus infections. To this end, TRIM6 was silenced in lung epithelial A549 cells using short interfering RNA (siRNA), and subsequently analyzed for cytokine and ISG induction upon SeV or influenza A virus (IAV; PR8 strain) infection (Figure 2). TRIM6 was silenced by more than 75% at the mRNA (Figures 2A and S2A) and protein levels (Figure S2B). SeV and IAV infection strongly induced IFN $\beta$  induction -albeit with different kinetics, which was attenuated by TRIM6<sup>si</sup> without an effect on pro-inflammatory TNF $\alpha$  expression (Figure 2B). ISGs including RIG-I, OAS1, MxA and ISG54 were all reduced by TRIM6<sup>si</sup> (Figure 2C), whereas viral RNA replication was increased (Figure 2D). Together, these data demonstrate that TRIM6 is required for efficient IFN and ISG induction and that its

depletion impairs the antiviral response as a result of attenuated IFN-I signaling, a phenotype reminiscent of that described for IKK $\epsilon$ -deficient mice (Tenover et al., 2007).

Immunoblot analyses showed that phosphorylation of IRF3 and IKK $\epsilon$  (a hallmark of activation) was reduced in TRIM6<sup>si</sup> cells upon infection with wild-type (WT) IAV or a recombinant virus (R38A/K41A) unable to effectively antagonize IFN induction (Figure 2E) (Talon et al., 2000). The effect of TRIM6 was not specific for RIG-I induced IFN $\beta$  induction, since TRIM6<sup>si</sup> also attenuated poly I:C-induced IFN $\beta$  and ISG54 mRNA expression (Figure 2F), as well as secreted IFN $\beta$  protein (Figure S2C) through MDA5. Consequently, the lower amounts of IFN $\beta$  production observed in TRIM6<sup>si</sup> cells attenuated downstream IFN signaling as indicated by a reduction of ISGF3 binding to the MxA, OAS1 and ISG15 promoters in an electrophoretic mobility shift assay (EMSA) (Figure 2G). These results indicate that TRIM6 is required for optimal signaling to induce IFN $\beta$  and ISGs during viral infection or dsRNA stimulation.

TRIM6 also contributes to the establishment of an efficient antiviral response against SeV (recognized by RIG-I) and Encephalomyocarditis virus (EMCV) (recognized by MDA5) (Figure 2H and I) in hDCs. At 6 h pi., IFN $\beta$  induction was significantly reduced in TRIM6<sup>si</sup> cells as compared to controls (Figure 2H, SeV  $p < 0.001$ , EMCV  $p < 0.003$ ; Figure 2I), whereas the levels of IL6 were not significantly affected (Figures 2H-I). SeV replication (assessed by gRNA RT-qPCR) was significantly increased in 3 out of 4 donors (Figure 2I;  $p < 0.001$ ) whereas all 4 donors showed significant increase in EMCV viral titers (Figures 2H-I;  $p < 0.001$ ). These data demonstrate that TRIM6 is critical for efficient IFN $\beta$  production and establishment of an antiviral state in relevant primary human cells.

### **TRIM6 interacts through its C-terminal SPRY domain with IKK $\epsilon$**

TRIM6 is required for optimal induction of IFN $\beta$ , but not NF- $\kappa$ B-dependent genes (Figures 1 and 2). We thus hypothesized that TRIM6 acts at the level of TBK-1, IKK $\epsilon$  or IRF3 and investigated whether TRIM6 interacts with any of these three candidates. Co-immunoprecipitation (coIP) studies demonstrated that TRIM6 efficiently interacted with IKK $\epsilon$  but not with the closely related kinase TBK-1 or the transcription factor IRF3 when co-expressed in HEK-293T cells (Figure 3A), suggesting that TRIM6 may be required for IKK $\epsilon$  activation. These results were confirmed for endogenous TRIM6 and IKK $\epsilon$  in primary hDCs upon LPS stimulation, SeV infection or IFN $\beta$  treatment (Figure 3B).

Subsequently, confocal microscopy revealed that TRIM6 localizes in punctate cytoplasmic bodies as previously reported (Reymond et al., 2001), whereas IKK $\epsilon$  exhibits a diffuse localization throughout the cytoplasm (Figure 3C). However, upon IKK $\epsilon$  and TRIM6 co-expression, a substantial fraction of the total IKK $\epsilon$  co-localized with TRIM6 and thus appears to be recruited to the cytoplasmic bodies where TRIM6 resides (Figure 3C). Endogenous IKK $\epsilon$  and TRIM6 also co-localized in hDCs, and this co-localization increased upon IFN $\beta$  treatment and SeV infection, further substantiating the relevance of the IKK $\epsilon$ -TRIM6 interaction in primary immune cells (Figure S3A).

To map the region of TRIM6 binding to IKK $\epsilon$ , we generated deletion mutants of TRIM6 expressing the RING domain, Bbox domain, or the C-terminal SPRY domain (Figure 3D),

and tested interaction with IKK $\epsilon$  by coIP. The C-terminal SPRY domain of TRIM6 by itself specifically interacted with IKK $\epsilon$  similar to full-length TRIM6, whereas a mutant lacking only the SPRY domain lost the ability to interact with IKK $\epsilon$  (Figure 3E). Furthermore, the SPRY domain of TRIM6 interacted *in vitro* with baculovirus-produced recombinant IKK $\epsilon$  protein (Figure S3B). Taken together these data indicate that TRIM6 interacts directly with IKK $\epsilon$  through its SPRY domain.

### TRIM6 is critical for the antiviral response mediated by the IFN $\beta$ signaling-IKK $\epsilon$ axis

Although IKK $\epsilon$  has been implicated in the signaling pathway to produce IFN-I, recent studies using IKK $\epsilon$ -deficient mice demonstrated a predominant role for IKK $\epsilon$  in the IFN signaling pathway by inducing a subset of IKK $\epsilon$ -dependent ISGs (Figure 4A) (Perwitasari et al., 2011; Tenover et al., 2007). Since TRIM6 interacted with IKK $\epsilon$ , we sought to determine the role of TRIM6 in the IFN-I signaling pathway.

We first determined whether TRIM6 enhances the induction of ISGs upon IFN treatment and if IKK $\epsilon$  is required for this effect (Figure 4A). To test this, TRIM6 was exogenously expressed in WT or *Ikk $\epsilon$ <sup>-/-</sup>* (the gene encoding IKK $\epsilon$ ) murine embryonic fibroblasts (MEFs). TRIM6 overexpression effectively enhanced the IFN $\beta$ -induced ISG54 promoter activity in WT MEFs but not in *Ikk $\epsilon$ <sup>-/-</sup>* cells (Figure 4B). This activity was not cell line-specific as TRIM6 was also able to enhance the IFN $\beta$ -induced ISG54 reporter activity in *Ddx58<sup>-/-</sup>* (the gene encoding RIG-I) MEFs (Figure 4B), which are only attenuated in their IFN induction, but not signaling. These results demonstrate that TRIM6 specifically requires IKK $\epsilon$  to induce ISGs. In line with these results, TRIM6<sup>si</sup> attenuated IKK $\epsilon$ -dependent ISG54 and OAS1 mRNA expression upon IFN $\beta$  treatment, whereas IKK $\epsilon$ -independently regulated ISG15 and IRF7 mRNA expression was not affected (Figure 4C). Moreover, depletion of TRIM6 enhanced IFN $\gamma$ -mediated expression of ISGs (Figure S4A), similar to the effects previously reported in *Ikk $\epsilon$ <sup>-/-</sup>* MEFs (Ng et al., 2011).

As previously reported, IKK $\epsilon$  underwent rapid activation by auto-phosphorylation on T501 upon IFN $\beta$  treatment of control A549 cells, which was impaired by TRIM6<sup>si</sup> (Figure 4D). In contrast, phosphorylation of STAT1 on Y701 and STAT2 on Y689 (which are IKK $\epsilon$ -independent) were unaffected by TRIM6<sup>si</sup> (Figure 4D), indicating that TRIM6 is specifically required for optimal IKK $\epsilon$  activation, but not for classic JAK1 and TYK2-mediated activation of STATs. As expected, TBK1 was not phosphorylated by IFN $\beta$  treatment and remained unaffected by TRIM6<sup>si</sup> (Figure S4B).

IKK $\epsilon$  has been previously shown to phosphorylate S708 on STAT1, which is required for IKK $\epsilon$ -dependent ISG induction (Figure 4A) (Perwitasari et al., 2011; Tenover et al., 2007). Consistent with a role of TRIM6 on the IKK $\epsilon$ -STAT1 arm of the IFN signaling pathway, IFN-induced S708 phosphorylation of STAT1 was impaired in TRIM6-silenced hDCs. In contrast, IKK $\epsilon$ -independent STAT1-Y701 phosphorylation was not affected (Figure 4E). Moreover, IKK $\epsilon$  auto-phosphorylation on T501 was also impaired in TRIM6<sup>si</sup> hDCs (Figure 4E), which is in agreement with the results in A549 cells (Figure 4D). An IKK $\epsilon$ -dependent role for TRIM6 was further substantiated by EMSA analysis, demonstrating that TRIM6<sup>si</sup> attenuated ISGF3 binding to the 'IKK $\epsilon$ -dependent' OAS1 promoter (Figure 4F).



We next determined the requirement of TRIM6 for conferring biologically relevant IFN-mediated antiviral activity to virus infection. TRIM6<sup>si</sup> in non-stimulated A549 cells only slightly increased the replication IAV expressing GFP (Figure 4G-H). In contrast, TRIM6<sup>si</sup> attenuated antiviral activity of IFN pre-treatment, which resulted in a ~ 3-fold increase in the fraction of virus-infected cells (~ 35%, Figure 4G-H). Taken together, these results demonstrate that TRIM6 is required for optimal activation of the IKK $\epsilon$  branch of the IFN-signaling pathway and is essential to establish an efficient antiviral state in cells.

### **IKK $\epsilon$ interacts with K48-linked poly-ubiquitin chains in cell culture and *in vivo***

TRIM6, like other TRIM family members, contains a RING domain, which has been shown to confer E3-Ub ligase activity (Meroni and Diez-Roux, 2005). Since we have shown that TRIM6 is important for IKK $\epsilon$  activity, we determined whether TRIM6 is involved in ubiquitination of IKK $\epsilon$ , the type of Ub linkage it synthesizes, and if this modification is important for IKK $\epsilon$  activity. To answer these questions, IKK $\epsilon$  and different Ub lysine mutants were exogenously expressed in the presence or absence of TRIM6 and the levels of IKK $\epsilon$ -associated Ub were assessed by CoIP. For these assays we used low amounts of IKK $\epsilon$  to avoid over-activation of the cells.

IKK $\epsilon$  pulled-down low levels of Ub in the absence of TRIM6 (Figure 5A, lane 4, IP). The amount of Ub that coIP with IKK $\epsilon$  was greatly enhanced by TRIM6 (Figure 5A, compare IP lane 5 and 6 to lane 4), suggesting that TRIM6 synthesizes poly-Ub forms which either covalently or non-covalently interact with IKK $\epsilon$ . These poly-Ub forms were predominantly K48-linked since coIP of a K48R Ub mutant with IKK $\epsilon$  was reduced as compared to other lysine mutants or WT Ub (Figure 5A, compare IP lane 12 to other lanes). In fact, poly-Ub chains formed exclusively through K48-linkage (all other lysines mutated to arginine) coIP more efficiently with IKK $\epsilon$  as compared to WT-Ub (Figure 5A, compare lane 14 to lane 6).

Importantly, poly-Ub chains did not coIP with IKK $\epsilon$  in the presence of a TRIM6 mutant lacking the C-terminal SPRY domain (Figure 5A, lane 15), which is required for TRIM6 binding to IKK $\epsilon$  (see Figure 3E), indicating that formation of a TRIM6-IKK $\epsilon$  complex is necessary for the IKK $\epsilon$ -Ub interaction. Endogenous K48-linked Ub chains also coIP with IKK $\epsilon$ , which was abolished in a TRIM6 E3 catalytic mutant (C15A; Figure 5B). In agreement, the same RING mutant failed to enhance the IFN signaling pathway (Figure 5C) or rescue ISRE reporter enhancement upon TRIM6<sup>si</sup> (Figure 5D). In line with these exogenous expression studies, association of endogenous IKK $\epsilon$  with K48-linked poly-Ub chains increased upon IFN $\beta$  treatment in control hDCs, whereas it was reduced in TRIM6<sup>si</sup> cells (Figure 5E). Together, these results indicate that TRIM6 requires its E3 activity for IKK $\epsilon$ -Ub association and enhancement of IFN signaling.

In an effort to ascertain that the TRIM6-IKK $\epsilon$ -poly-Ub interaction was physiologically relevant and occurs *in vivo*, we investigated whether these three proteins interacted by coIP assays in the lungs of mice treated with universal type I IFN (Figure 5F). Intranasal (i.n.) IFN treatment resulted in rapid and transient STAT1 phosphorylation at Y701 (WCE, 45min-2hr), followed by STAT1 phosphorylation at S708 (WCE, 2-6hr). Consistent with the proposed role of IKK $\epsilon$  in STAT1-S708 phosphorylation, activation of IKK $\epsilon$  (T501 phosphorylation) correlated with STAT1-S708 phosphorylation. Consequently, coIP of

poly-Ub chains and TRIM6 with IKK $\epsilon$  also correlated with IKK $\epsilon$ -T501 and STAT-S708 phosphorylation (Figure 5F).

To further demonstrate that TRIM6 is required for the formation of IKK $\epsilon$ -Ub complexes and establishment of an efficient antiviral response *in vivo*, TRIM6 expression was silenced in the lungs of mice using peptide-conjugated phosphorodiamidate morpholino oligomers (PPMO) (Abes et al., 2006). In line with our results from hDCs and cell lines, the designed TRIM6 PPMOs efficiently and specifically downregulated TRIM6 protein levels in MEFs resulting in a reduction of ISG54 protein expression upon IFN $\beta$  treatment (Figure S5A). To test the effects of PPMO-mediated TRIM6<sup>si</sup> *in vivo*, we first determined the kinetics of IAV infection *in vivo*. Mice not treated with PPMO were infected i.n. with IAV for up to five days, and analyzed for TRIM6, IKK $\epsilon$  and poly-Ub interaction in their lungs by coIP at different times p.i. (Figure S5B). Viral loads peaked at 3 d p.i. and remained high until 5 d p.i. (Figure S5C). Viral replication in the lungs of infected mice correlated with increased mRNA levels of M1 viral RNA, IFN $\beta$ , TRIM6 and the IFN-stimulated gene ADAR1 (Figure S5D). The protein levels of TRIM6, Ub and IKK $\epsilon$  increased in the lungs of infected mice as compared with controls (Figure S5E, WCE). Furthermore, IKK $\epsilon$  interaction with TRIM6 and Ub was detected as early as 1 d p.i. and increased in a time-dependent manner (Figure S5E, IP).

We next assessed the effects of PPMO-mediated TRIM6<sup>si</sup> on viral load, cytokine and ISG induction, and IKK $\epsilon$ -Ub association at early time points p.i., before the peak-level of viral titer is typically observed in the lungs of this mouse model (Figure S5F). Treatment with PPMOs resulted in ~50% reduction in TRIM6 mRNA and protein compared to non-targeting PPMO control in the lungs of infected and non-infected mice (Figure 5H, WCE and S5G). Reduction in TRIM6 expression in the lungs of mice correlated with increased viral load, as assessed by plaque assay (Figure 5G), RT-qPCR for viral M1 mRNA (Figure S5G), and immunoblot for viral nucleoprotein (NP) and NS1 protein (Figure 5H, WCE). The effects of TRIM6 silencing mirrored the phenotype previously observed in *Ikkbe*<sup>-/-</sup> mice upon IAV infection (Tenoever et al., 2007). Namely, although the levels of IFN $\beta$  mRNA were higher in the lungs of TRIM6-silenced mice, induction of the IKK $\epsilon$ -dependent ISGs ADAR1 and IFI44 was reduced as compared to controls (Figure S5G), consistent with defective IFN-I signaling. In contrast, the induction of pro-inflammatory cytokines TNF $\alpha$  and IL-6 as well as IL-12p40 (Figure S5G) was unaffected, indicating that TRIM6-silencing *in vivo* specifically affects the IFN-I-IKK $\epsilon$  axis, consistent with our observations in human DCs.

Importantly, the formation of IKK $\epsilon$ -Ub complexes and IKK $\epsilon$  auto-phosphorylation were reduced in TRIM6<sup>si</sup> lungs as compared to controls (Figures 5H and S5H), indicating that TRIM6 is required for IKK $\epsilon$  activation *in vivo*. In support of this, STAT1-S708 phosphorylation was also reduced in TRIM6<sup>si</sup> lungs (Figure 5H, WCE). In contrast, STAT1 induction (an IKK $\epsilon$ -independent ISG) and STAT1-Y701 phosphorylation (which is IKK $\epsilon$ -independent) were not affected in TRIM6<sup>si</sup> lungs (Figure 5H, WCE) indicating that TRIM6 specifically acts in the IKK $\epsilon$  axis of the IFN-I signaling *in vivo* and confirms our results obtained in cell lines and human DCs.

Taken together, these data indicate that TRIM6 facilitates Ub chain synthesis required for efficient IKK $\epsilon$  function in IFN-I signaling and demonstrate that TRIM6 is required for establishment of an efficient antiviral response in cultured cells and *in vivo*.

### **TRIM6 facilitates the synthesis of unanchored ubiquitin chains that interact with IKK $\epsilon$ and are required for efficient type I IFN signaling**

The previous analyses did not determine whether IKK $\epsilon$  is covalently modified by Ub or whether it interacts non-covalently with ubiquitinated proteins or unanchored Ub chains. Furthermore, previous studies have also found Ub associated with IKK $\epsilon$  (Friedman et al., 2008; Parvatiyar et al., 2010), but did not address whether these Ub chains were unanchored or covalently attached. To distinguish between these possibilities, a denaturing pull-down analysis of his-tagged Ub co-expressed with IKK $\epsilon$  was performed (Figure S6A). In contrast to the results from the above-described coIP under native conditions, we did not detect ubiquitinated IKK $\epsilon$  under denaturing pull-down conditions (Figure 6A), while ubiquitinated TRIM6 was readily detected, confirming the validity of the assay. Also, higher migrating – potentially ubiquitinated- forms of IKK $\epsilon$  were not detected in the WCE (Figure 6A). Together, these data suggested that IKK $\epsilon$  might interact non-covalently with TRIM6-synthesized unattached poly-Ub chains or other TRIM6-ubiquitinated cellular proteins.

As unanchored K63 Ub chains have recently been recognized as significant signaling mediators (Pertel et al., 2011; Xia et al., 2009; Zeng et al., 2010), we set out to determine whether the TRIM6-synthesized Ub moieties interacting with IKK $\epsilon$  were in fact unanchored Ub chains. To this end we utilized co-expression of isopeptidase T/USP5 (IsoT), which has been well-established to specifically degrade unanchored Ub through recognition of its exposed C-terminal diglycine residues, while not deconjugating attached Ub chains (Figure S6B) (Reyes-Turcu et al., 2006; Zeng et al., 2010). Indeed, in agreement with our hypothesis, co-expression of IsoT decreased the amount of TRIM6-synthesized multimeric Ub interacting with IKK $\epsilon$  in a dose-dependent manner (Figure 6B, left panel). Likewise, treatment of isolated IKK $\epsilon$ -Ub complexes with recombinant IsoT *in vitro* also reduced the amount of poly-Ub chains that coIP with IKK $\epsilon$  (Figure S6C). In contrast, IsoT expression did not have a major effect on multimeric Ub co-immunoprecipitated with JAK1, which was used as a control (Figure 6B, right panel). This suggests that JAK1-associated Ub chains are primarily covalently attached, whereas the poly-Ub chains associated with IKK $\epsilon$  are unanchored and their synthesis is mediated by TRIM6.

Furthermore, IsoT co-expression reduced the TRIM6-dependent ISG54 reporter activation almost to the level of the control without TRIM6 (Figure 6C and 6D), suggesting that unanchored Ub chains are important for TRIM6-dependent IKK $\epsilon$  enhancement. In agreement, cleavage of both anchored and unanchored poly-Ub chains by co-expression of the ovarian tumor (OTU) domain of Crimean Congo hemorrhagic fever virus (CCHFV) large (L) protein (Frias-Staheli et al., 2007), also negated TRIM6-mediated IKK $\epsilon$  activation of the ISG54 promoter (Figure 6D). In contrast, a non-catalytic control mutant lacking this activity did not (Figure 6D).

We next determined whether the formation of the IKK $\epsilon$ -Ub complex *in vivo* requires IFN signaling during infection and whether the Ub chains formed *in vivo* are indeed unanchored.



To this end, WT and *Ifnar1*<sup>-/-</sup> mice were infected i.n. with IAV for up to five days, and analyzed for IKKε and poly-Ub interaction in their lungs by coIP at different times p.i.. In agreement with the results described above, K48-linked poly-Ub chains and TRIM6 coIP with IKKε and increased over time in the lungs of virus infected WT mice, but not *Ifnar1*<sup>-/-</sup> mice, indicating that the formation of this complex is IFN-I signaling dependent (Figure S6D). Importantly, the poly-Ub chains bound to IKKε in WT mice were unanchored since they were degraded by *in vitro* treatment with recombinant IsoT (Figure S6D; compare ‘day 3 WT’ in lane 4 with ‘day 3 WT + IsoT’ in lane 11).

Finally, to demonstrate that IKKε interacts directly with unanchored K48-linked Ub chains, a cell-free system with baculovirus-expressed recombinant IKKε purified from insect cells was established and used to determine IKKε binding to recombinant Ub chains *in vitro* (Figure S6E). IKKε interacted with K48-linked (lanes 9-10), but not K63-linked poly-Ub (lane 11) or K48-K63 mixed-linkage tetra-Ub (lane 12). Binding of IKKε to Ub required a minimum of four Ub units since di- or tri-Ub did not co-immunoprecipitate with IKKε under these conditions (Figure S6E).

Taken together, these results show that i) IKKε interacts directly with unanchored K48-linked poly-Ub chains, ii) TRIM6 enhances IKKε interaction with these unanchored K48-linked Ub chains, and iii) these Ub chains are important for TRIM6-IKKε-dependent ISG induction.

### **IKKε is recruited to TRIM6-ubiquitin-rich bodies**

Next, it was addressed whether TRIM6 and IKKε co-localize with Ub in the cell by confocal microscopy. TRIM6 localized into defined cytoplasmic dots (Figure 6E), in agreement with previous findings ((Reymond et al., 2001) and Figure 3C). Exogenously expressed Ub alone exhibited weak staining distributed throughout the cell. This Ub staining increased when co-expressed with TRIM6 and extensively co-localized with the punctate cytoplasmic TRIM6 bodies (Figure 6E). Similarly, IKKε alone localized diffusely throughout the cytoplasm (Figures 6F and 3C), and re-localized into the same compartment as both TRIM6 and Ub upon co-expression of TRIM6 (Figure 6F).

Exogenous IsoT expression disrupted the TRIM6 punctate structures, without affecting TRIM6-IKKε co-localization (Figure S6F), as well as *in vitro* treatment with IsoT specifically degraded the unanchored poly-Ub chains associated with TRIM6, while not having an effect on covalently ubiquitinated TRIM25 used as a control (Figure S6G). Together these data suggest that unanchored Ub chains are essential for the formation of cytoplasmic “TRIM-Ub-rich bodies” but not for TRIM6-IKKε interaction. They indicate that i) TRIM6 enhances the synthesis of Ub forms in defined sub-cellular structures and ii) IKKε interacts with TRIM6 and requires TRIM6-mediated Ub synthesis for its activity.

### **TRIM6 and the E2 conjugase UbE2K cooperatively synthesize unanchored K48-linked ubiquitin chains required for IKKε activation**

To demonstrate that TRIM6 can synthesize K48-Ub chains directly mediating IKKε activation, a TRIM6 *in vitro* ubiquitination assay was established. Ub conjugation requires

the activity of E1 activating, E2 conjugating and E3 ligating enzymes. Since the E2 enzyme that couples TRIM6 to Ub conjugation had yet to be determined, first an *in vitro* screen with twenty-nine common E2-ligases was performed for their ability to synthesize poly-Ub chains in the presence of TRIM6.

By these means we identified three E2 enzymes (Ube2K, CDC34, Ube2G2; data not shown) previously reported to facilitate K48-linked Ub chain synthesis (Chen and Pickart, 1990; Petroski and Deshaies, 2005; Shin et al., 2011), which were tested in knockdown experiments for their requirement in IFN $\beta$ -dependent ISG induction. Only Ube2K-silencing in A549 cells resulted in a decrease of ISG54 mRNA induction upon IFN stimulation as compared to control cells (Figure 7A). In contrast, silencing of CDC34 or Ube2G2 did not attenuate ISG54 mRNA induction, or even increased it (Figure 7A), despite similar knockdown (>80% data not shown).

The Ube2K conjugase is known to elongate mono-Ub to produce K48-linked poly-Ub chains (Wilson et al., 2011), and has been reported to have the ability to synthesize unanchored poly-Ub chains in the absence of an E3 ligase *in vitro* (Chen and Pickart, 1990). Thus, as anticipated, K48-linked poly-Ub chains were produced in a Ube2K dose-dependent manner (Figure 7B). Under these conditions (at high concentrations of Ube2K and high amounts of synthesized K48 poly-Ub) IKK $\epsilon$  auto-phosphorylation was detected on T501 (Fig 7B), demonstrating that Ube2K can mediate K48 Ub chain synthesis and IKK $\epsilon$  activation. Moreover, K48-linked poly-Ub chain production and IKK $\epsilon$  activation by the lowest concentration of Ube2K (0.025  $\mu$ M) were enhanced by TRIM6 in a dose-dependent manner (Figure 7C), indicating that TRIM6 in combination with Ube2K acts as an E3 ligase driving the synthesis of K48 poly-ubiquitin. IKK $\epsilon$  auto-phosphorylation was specific since T501 was not phosphorylated in the presence of an inactive IKK $\epsilon$  kinase mutant (K38A; Figure S7A). As expected, poly-Ub synthesis was completely dependent on ATP and the presence of Ube2K, but not IKK $\epsilon$  (Figure 7C). In addition, these poly-Ub chains were exclusively K48-linked since they were not produced from a K48R mono-Ub mutant substrate (Figure S7A). Conversely, chain synthesis of a Ub mutant with all lysines mutated except for K48 (K48only) was enhanced.

Next, IKK $\epsilon$  was incubated *in vitro* with purified, recombinant poly-Ub chains after which IKK $\epsilon$  activation was determined. Only a mixture of K48-linked poly-Ub chains (2-7 Ub molecules/chain) activated IKK $\epsilon$  T501 auto-phosphorylation in a dose-dependent manner, whereas similar amounts of K63-linked chains did not (Figure 7D). This further demonstrates that specifically K48-linked poly-Ub chains -such as synthesized by Ube2K and TRIM6- activate IKK $\epsilon$ .

We next confirmed that K48 poly-Ub-activated IKK $\epsilon$  has indeed increased kinase activity towards its *bona fide* target STAT1. K48- but not K63-linked poly-Ub chains reproducibly increased purified STAT1 phosphorylation by IKK $\epsilon$  in a dose-dependent manner (Figure 7E). Importantly, longer K48-linked poly-Ub chains (mixture of 2-16 Ubs/chain), which putatively more closely resemble the high molecular forms of Ub found *in vivo* (Figure 5F, S5E and S6D), induced even higher levels of STAT1 phosphorylation, as well as increased

IKK $\epsilon$  auto-phosphorylation (Figure 7F). Again, this was specific to IKK $\epsilon$  as no phosphorylation was detected with the IKK $\epsilon$  K38A mutant.

These results were substantiated by investigating whether isolated TRIM6-synthesized endogenous, unanchored poly-Ub chains could facilitate IKK $\epsilon$ -dependent phosphorylation of STAT1 *in vitro*. To this end, endogenous Ub chains bound to IKK $\epsilon$  were isolated as previously described for RIG-I (Zeng et al., 2010) (Figure S7B). In agreement with our other data, Ub chains isolated from IKK $\epsilon$  alone, limitedly increased STAT1 phosphorylation (Figure 7G; compare lanes 1 and 2). Strikingly, endogenous Ub chains isolated from IKK $\epsilon$  expressed in the presence of TRIM6, even further enhanced IKK $\epsilon$ -dependent STAT1 phosphorylation within the limited experimental window (Figure 7G; compare lanes 2 and 4). Moreover, the poly-Ub-mediated increase in STAT1 phosphorylation was abrogated upon IsoT treatment, confirming that the enhancement of STAT1 phosphorylation depends on unanchored poly-Ub chains (Figure 7G; compare lanes 2 with 3, and 4 with 5).

Finally, to better understand the mechanism of IKK $\epsilon$  activation by TRIM6 and poly-Ub chains, we mapped the region of IKK $\epsilon$  required for interaction with TRIM6 and Ub by coIP using truncated forms of IKK $\epsilon$  (Figure S7C). TRIM6 and poly-Ub chains interacted more efficiently with the kinase domain and IKK $\epsilon$  mutants lacking their C-terminal CCDs (Figure S7D). However, TRIM6 interacted less efficiently with a shorter form of IKK $\epsilon$  expressing both the kinase domain and the Ub-like domain (UBLD), suggesting that the UBLD could fold back on the kinase domain as previously reported (Ikeda et al., 2007), thereby interfering with TRIM6 binding to the IKK $\epsilon$  kinase domain. Furthermore, residue S172 in the IKK $\epsilon$  kinase domain was critical for interaction with TRIM6 and Ub (Figure S7D, last lane). In line with these observations, the IKK $\epsilon$  S172A mutant did not activate the ISG54 promoter in a reporter assay (Figure S7E). In addition, TRIM6 did not enhance the IKK $\epsilon$  S172A-dependent ISG54 reporter activity as compared to TRIM6 alone or WT-IKK $\epsilon$  alone (Figure S7F).

Since unanchored K48 Ub chains induced transactivation of IKK $\epsilon$  (Figure 7D), we hypothesized that TRIM6-synthesized poly-Ub chains are important for IKK $\epsilon$  oligomerization and thus transactivation by binding to the kinase domain of IKK $\epsilon$ . In line with this hypothesis, *in vitro* data indicated that IKK $\epsilon$  runs as a dimer under native conditions (Figure S7G) and addition of K48-linked poly-Ub chains increased the oligomerization of IKK $\epsilon$  with the strongest effect obtained in the presence of long K48-linked poly-Ub chains (Figure S7G). Thus, K48-linked poly-Ub chains promote oligomerization of IKK $\epsilon$  suggesting that oligomerization is important for transactivation.

### TRIM6 interacts with the JAK1 kinase

It remains to be determined how TRIM6 itself is regulated at the detailed molecular level. A possible mechanism could entail direct TRIM6 activation by the JAK1 and TYK2 kinases downstream of the IFN-I receptor. In support of this possibility, JAK1 interacted with TRIM6 and induced TRIM6 tyrosine phosphorylation (Figures S7H and S7I). Although the nature of this TRIM6 phosphorylated form remains to be determined, it is tempting to speculate that TRIM6 may be activated by JAK1 after IFN treatment.

Taken together, our results demonstrate that TRIM6 and Ube2K cooperate in the synthesis of K48-linked unanchored poly-Ub chains, which activate IKK $\epsilon$  for STAT1 phosphorylation (Figure S7J). The IKK $\epsilon$ -targeted phosphorylation site on STAT1 (S708) promotes antiviral activity through induction of a subset of ISGs. This phosphorylation is distinct from IKK $\epsilon$ -independent STAT1-Y701 phosphorylation mediated by JAK1 and TYK2, which drives IKK $\epsilon$ -independent ISG expression (Figure S7J).

## Discussion

In this study we demonstrate that the Ub E3 ligase TRIM6 is required for efficient IFN-I signaling and establishment of an antiviral state by synthesis of K48-linked unanchored poly-Ub chains, which activate IKK $\epsilon$  for STAT1 phosphorylation. Our findings indicate that these Ub chains play a role in both whole cell context and *in vivo*. This is supported by four lines of evidence: i) expression of IsoT, which specifically degrades unanchored Ub, reduced IFN-mediated ISG induction in cells, ii) knockdown of the E2-Ub ligase Ube2K, which is known to synthesize K48-linked poly-Ub chains (Chen and Pickart, 1990; Wilson et al., 2011), attenuated ISG expression upon IFN stimulation, iii) poly-Ub chains interact with IKK $\epsilon$  in mouse lungs during viral infection in an IFN-I-dependent manner and iv) the formation of IKK $\epsilon$ -Ub complexes is reduced in TRIM6<sup>si</sup> mouse lungs upon viral infection.

Importantly, TRIM6<sup>si</sup> not only impaired IFN signaling, but also impaired IFN $\beta$  production upon TLRs and RLRs stimulation in primary human DCs and human cell lines, although this was not the case in the lungs of IAV-infected mice. One possible explanation for this discrepancy could be differences in TRIM6 function in human and mouse. Alternatively, TRIM6 may have cell type specific effects or may act at multiple levels in signaling pathways of the IFN-I system. Moreover, increased IFN production in TRIM6<sup>si</sup> mice may be a reflection of increased viral replication as a result of diminished antiviral response. In addition, any defect on IFN production in TRIM6<sup>si</sup> cells in the lung may have been obscured by IFN produced from other cells in the lung that were not subject to TRIM6<sup>si</sup>, as for example, infiltrating cells.

Although it is clear that IKK $\epsilon$  phosphorylates IRF3 and IRF7 for IFN induction (Sharma et al., 2003), production of IFN-I was not reduced in *Ikk $\epsilon$ <sup>-/-</sup>* mice upon IAV infection (Tenoever et al., 2007). However, studies have suggested that TBK-1 and IKK $\epsilon$  play a redundant role in IFN induction (Hemmi et al., 2004). Therefore it is still plausible that TRIM6 may also play a role by activating IKK $\epsilon$  for IFN $\beta$  production in specific cell types, or under specific conditions. Alternatively, TRIM6 may have other signaling partners in addition to IKK $\epsilon$  for IFN induction, as it has been shown for other TRIMs (Rajsbaum et al., 2014). The specific role of TRIM6 in the IFN $\beta$  induction pathway will need to be determined in future studies.

In our immunofluorescence experiments, TRIM6, IKK $\epsilon$  and Ub co-localized in punctate cytoplasmic bodies. These structures, which have also been observed for other TRIMs, seem to be independent sub-cellular compartments since they do not co-localize with any markers for known cellular structures (Reymond et al., 2001). As we observed a dramatic increase in Ub staining in these TRIM6-containing bodies, we speculate that these structures may be

“Ub factories” where other target proteins -such as IKK $\epsilon$ - can be recruited during signaling. These structures required an intact TRIM6 coiled-coil region (data not shown, and (Reymond et al., 2001)), and formation of poly-Ub chains by TRIM6, suggesting that oligomerization is essential for their assembly. These sub-cellular structures may be an important compartmentalization mechanism in the cell.

Our data suggest that TRIM6 could be activated upon IFN stimulation by the JAK1 kinase by binding and phosphorylating TRIM6. However, TRIM6 activation may also involve ubiquitination by a yet unidentified E3-ligase since covalent K63-linked ubiquitination of both WT TRIM6 and its E3 ligase C15A mutant was detected.

In conclusion, our study identified TRIM6, Ube2K and K48-linked poly-Ub chains as novel players critical for the IKK $\epsilon$  branch of the IFN-I signaling pathway and subsequent establishment of a protective antiviral response. Our study provides a framework for future work on the TRIM family of proteins, as well as the mechanism of kinase activation by unanchored K48-linked poly-Ub chains related to antiviral immunity. Whereas we report a unique function of unanchored K48-poly-Ub in kinase activation, it might be possible that these molecules also participate in other signaling process, as is the case for unanchored K63-poly-Ub (Pertel et al., 2011; Xia et al., 2009; Zeng et al., 2010). Free poly-Ub is therefore emerging as a secondary messenger that participates in regulation of signaling and further studies are needed to fully elucidate cellular processes that are controlled by the synthesis of unanchored Ub chains.

## Experimental Procedures

### *In vitro* ubiquitination assay and activation of IKK $\epsilon$

Ubiquitination assays *in vitro* were performed using the Ub conjugation initiation kit (Boston Biochem). In brief, a mix was prepared containing the E1 enzyme, Ub, and reaction buffer in the presence or absence of the E2 ligase Ube2K (Boston Biochem) (0.02-0.5mM) and the presence or absence of FLAG purified IKK $\epsilon$  (0.1  $\mu$ l) and HA-TRIM6 purified from HEK-293T cells with HA-beads (0.05-0.5  $\mu$ l). The reaction was initiated with addition of 1mM Mg-ATP and stopped after 1h at 30°C by addition of Laemmli sample buffer (BioRad) containing  $\beta$ -Mercaptoethanol and boiled for 5 min. Poly-Ub and IKK $\epsilon$  auto-phosphorylation was detected by IB with  $\alpha$ -pIKK $\epsilon$ (T501) (Novus Biologicals).

### Isolation and analysis of endogenous poly-ubiquitin chains

We followed a previously described protocol to isolate chains from RIG-I(2CARD) used by Zeng *et.al.* (Zeng et al., 2010). For detail information see Supplemental Information.

### siRNA-mediated gene targeting

Transient gene targeting of endogenous TRIM6 in A549 or HEK-293T cells, seeded in 24-well plates (30,000 cell/well), was achieved by transfection of 10 picomol of non-targeting control or an siRNA specific for TRIM6 (see supplemental information for sequences) with RNAiMAX (Invitrogen) according to the manufacturer's instructions.



### TRIM6 gene targeting in human DCs with shRNA lenti-viral vectors

Peripheral blood mononuclear cells were isolated from buffy coats of healthy human donors by Ficoll density gradient centrifugation (Histopaque, Sigma Aldrich) as previously described (Fernandez-Sesma et al., 2006). For additional details see Supplemental Information. All human research protocols for this work have been reviewed and approved by the Institutional Review Board of the Mount Sinai School of Medicine.

### *In vitro* oligomerization assay and native PAGE

Purified FLAG-IKK $\epsilon$  protein was incubated with Ub chains in the presence or absence of 1mM of ATP and 2 mM MgCl<sub>2</sub> in 50 mM Tris, 150 mM NaCl, 1 mM DTT buffer at 37°C for 15 minutes to allow formation of complexes. Reactions were stopped by addition of 1x Native PAGE sample buffer (Life) and subjected to native PAGE on a 3-12% Bis-Tris gel (Life), transferred on to PVDF membranes, fixed with 8% acetic acid in H<sub>2</sub>O and immunoblotting was performed using anti-FLAG antibodies.

### TRIM6 gene targeting *in vivo* using PPMOs

The peptide-conjugated phosphorodiamidate morpholino oligomers (PPMO) were produced by previously published methods (Abes et al., 2006). All mouse experiments were carried out in accordance with institutional Animal Care and Use Committee (IACUC) guidelines, and have been approved by the IACUC of Icahn School of Medicine at Mount Sinai. Balb/c mice were anesthetized with ketamine, followed by i.n. administration with 100  $\mu$ g (approximately 5 mg/kg) PPMO (in 40  $\mu$ l of PBS). After 24 hr, PPMO treatment was repeated. After 48 h from the first PPMO administration, mice were infected i.n. with 1000 PFU of influenza virus (strain A/Puerto Rico/8/1934 H1N1 [PR8]). At 24 and 48 h.p.i. mice were euthanized and the lungs were collected and divided into 3 equivalent portions, followed by homogenization and subsequent plaque assay, real time PCR analysis and coIP studies. For more details see Supplemental Information.

### Statistical Analysis

Statistical analysis was performed using Prism (Version 5.0, GraphPad Software, San Diego California USA). Student's paired t-test, or in defined cases two-way ANOVA with Bonferroni post-test were used. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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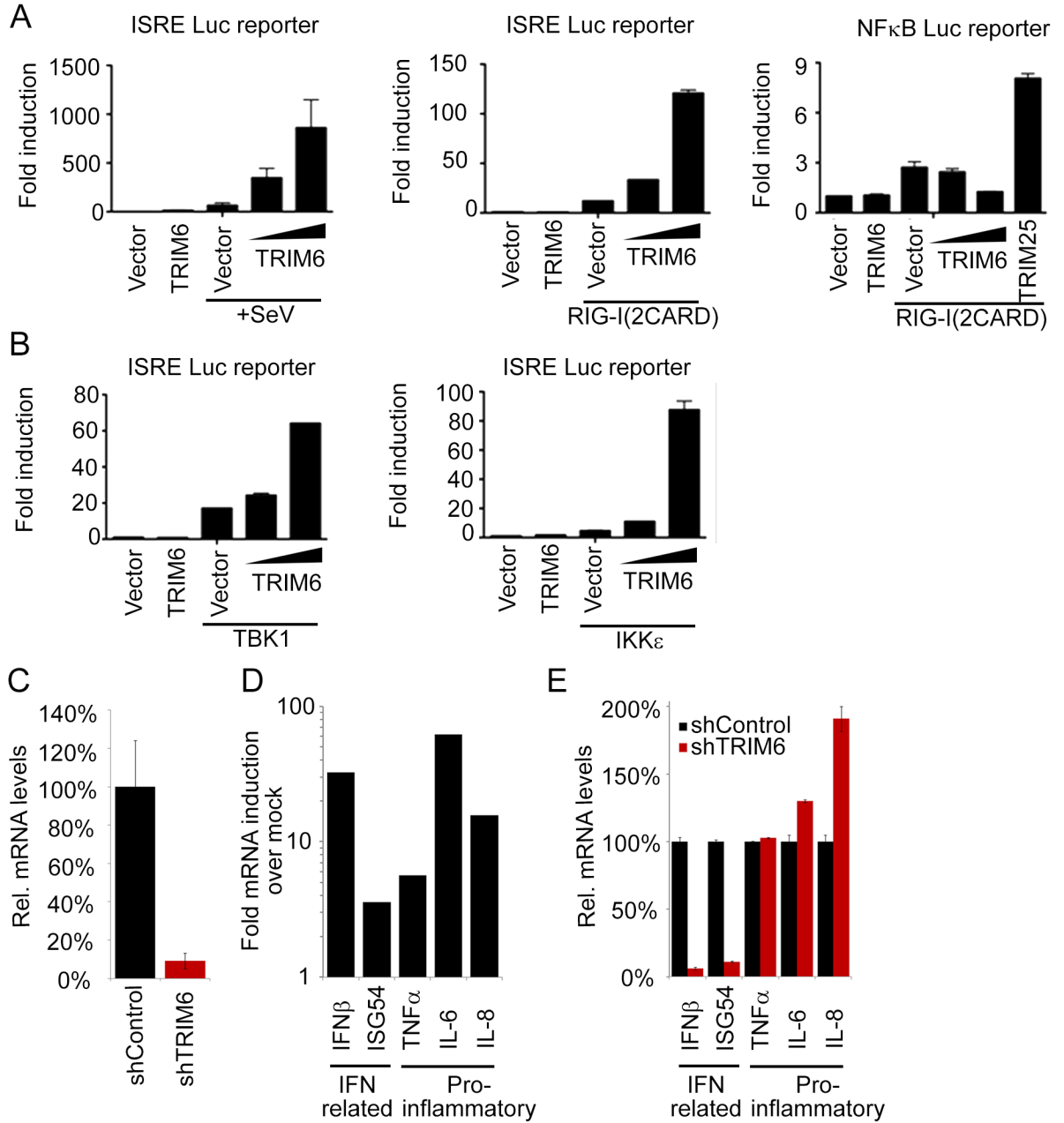
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### Highlights

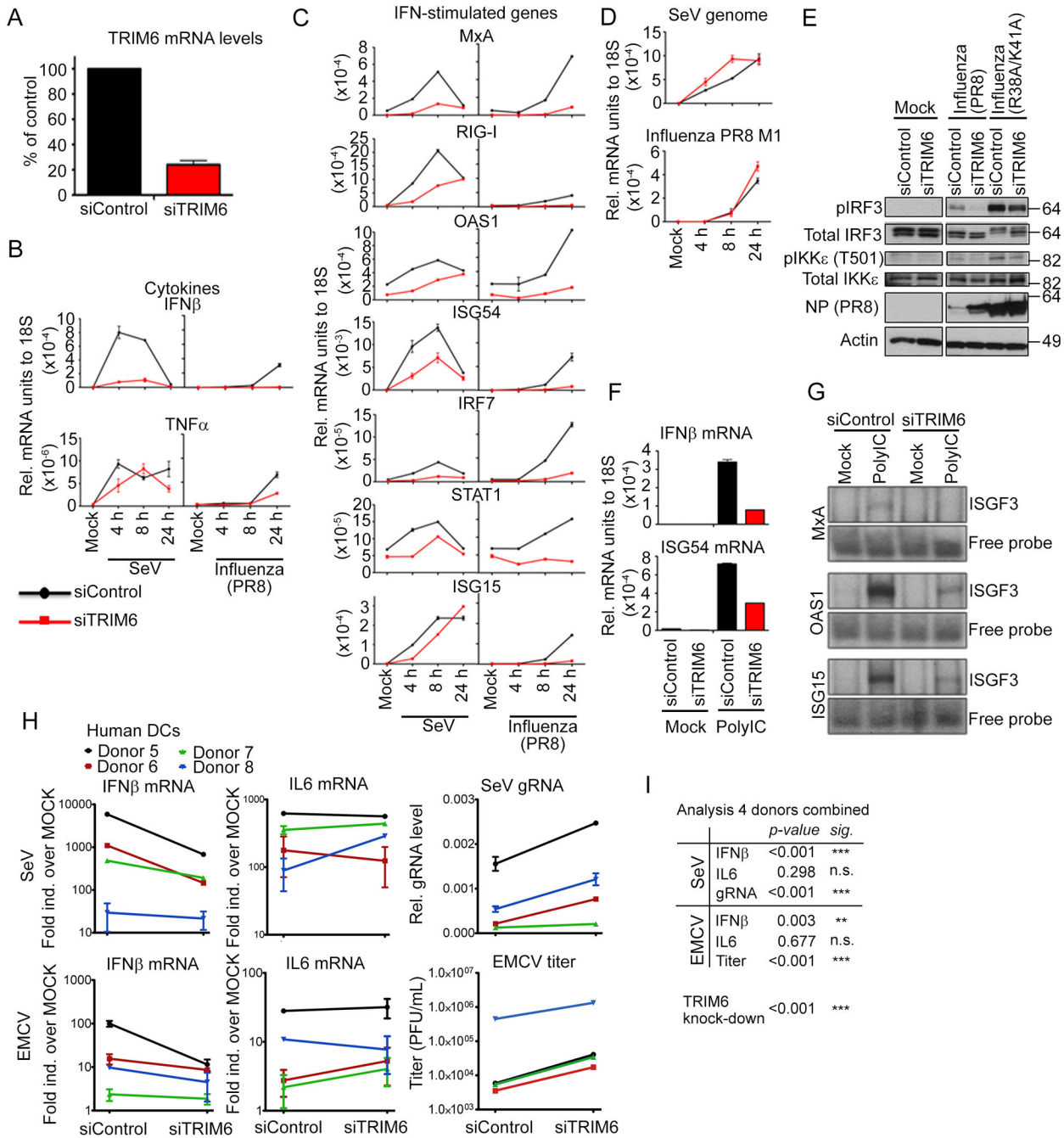
- TRIM6 plays a critical role in innate immune activation
- TRIM6 and the E2 conjugase UbE2K synthesize unanchored K48-linked ubiquitin chains
- Unanchored K48-linked ubiquitin chains activate the IKK $\epsilon$  kinase
- TRIM6 couples IFN $\alpha$ R engagement to activation of the IKK $\epsilon$  kinase for ISG induction





**Figure 1. TRIM6 enhances induction of IFN $\beta$  but not NF- $\kappa$ B-dependent cytokines**

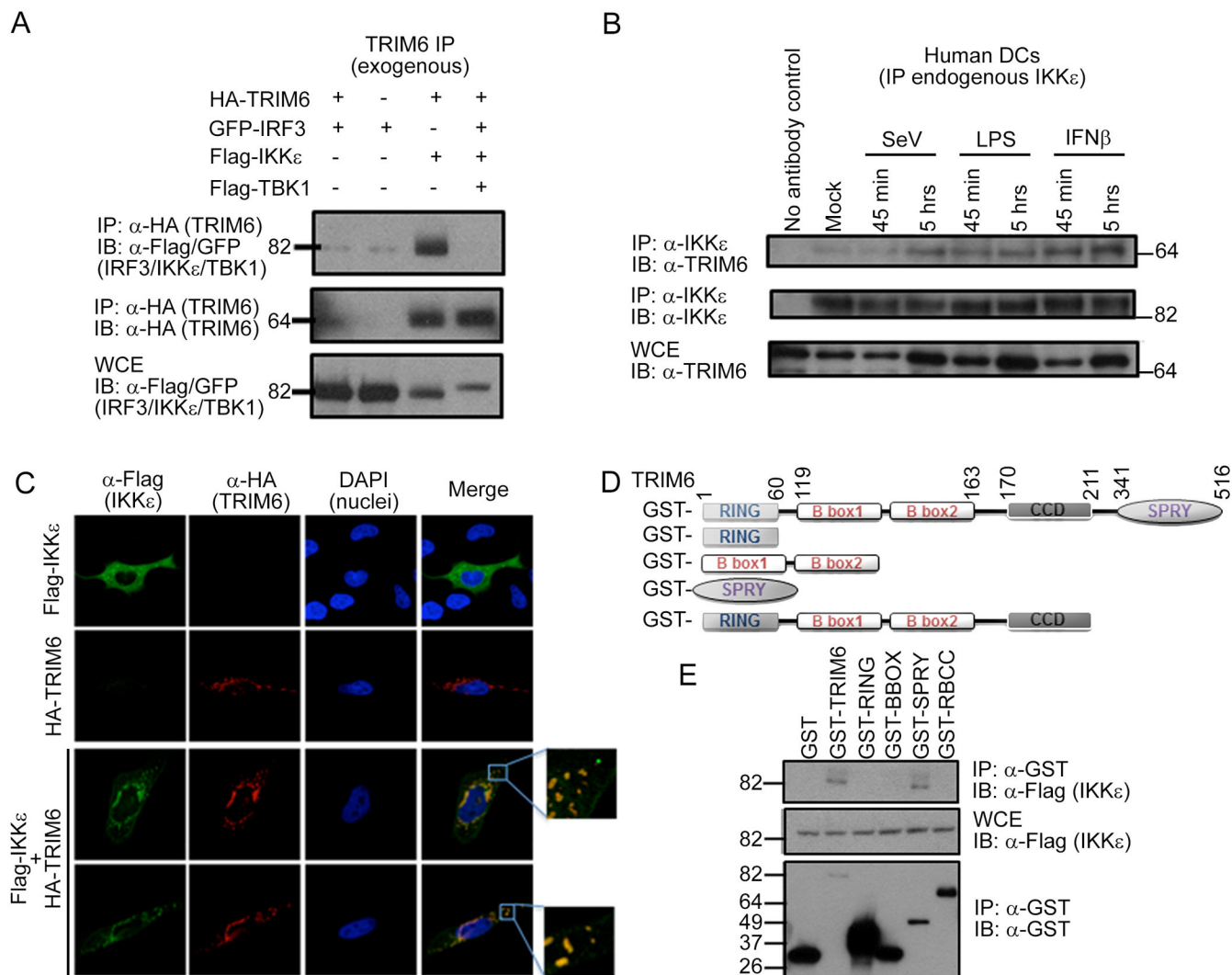
(A-B) HEK-293T cells were transfected with the ISG54 IFN-stimulated responsive element (ISRE) or NF- $\kappa$ B reporter plasmid together with TRIM6 or TRIM25 and stimulating plasmids or SeV as indicated, followed by luciferase assay. Data are representative of 3 independent experiments and depicted is the mean  $\pm$  SD (n=3). (C-E) hDCs transduced with lentiviruses expressing TRIM6 shRNAs were stimulated with LPS. At 2 h p.t. cells were harvested for RT-qPCR and plotted as percentage of control or fold induction over mock-induced samples. See also Figure S1.



**Figure 2. TRIM6 is required for efficient induction of IFNβ and ISGs**

(A-G) A549 cells were transfected with a control or TRIM6-specific siRNAs. (A-E) Forty h p.t., cells were mock-treated or infected with SeV (10 HAU/ml), IAV (PR8; 2 PFU/cell), (E) a recombinant NS1 RNA binding mutant virus (R38A/K41A), or (F-G) transfected with dsRNA poly (I:C). RNA levels were quantified by RT-qPCR. (E) At 8 h p.i. with IAV, cells were harvested for immunoblot (IB). (F) At 8 h p.t. with poly I:C, cells were harvested for RT-qPCR or (G) (4 h p.t.) EMSA analysis of ISGF3 binding to ISG promoters. (H) hDCs transduced with lentiviruses expressing TRIM6 shRNAs were infected with SeV or EMCV.

Cells were harvested at 6 h p.i for RT-qPCR analysis or at 24 h p.i for virus titration by RT-qPCR or plaque assay. Values are plotted as fold induction over mock samples. Data represent mean  $\pm$  SD. **(I)** The effect of knockdown for all four donors combined was determined by two-way ANOVA. \*\*p < 0.01; \*\*\*p < 0.001, N.S. (not significant). See also Figure S2



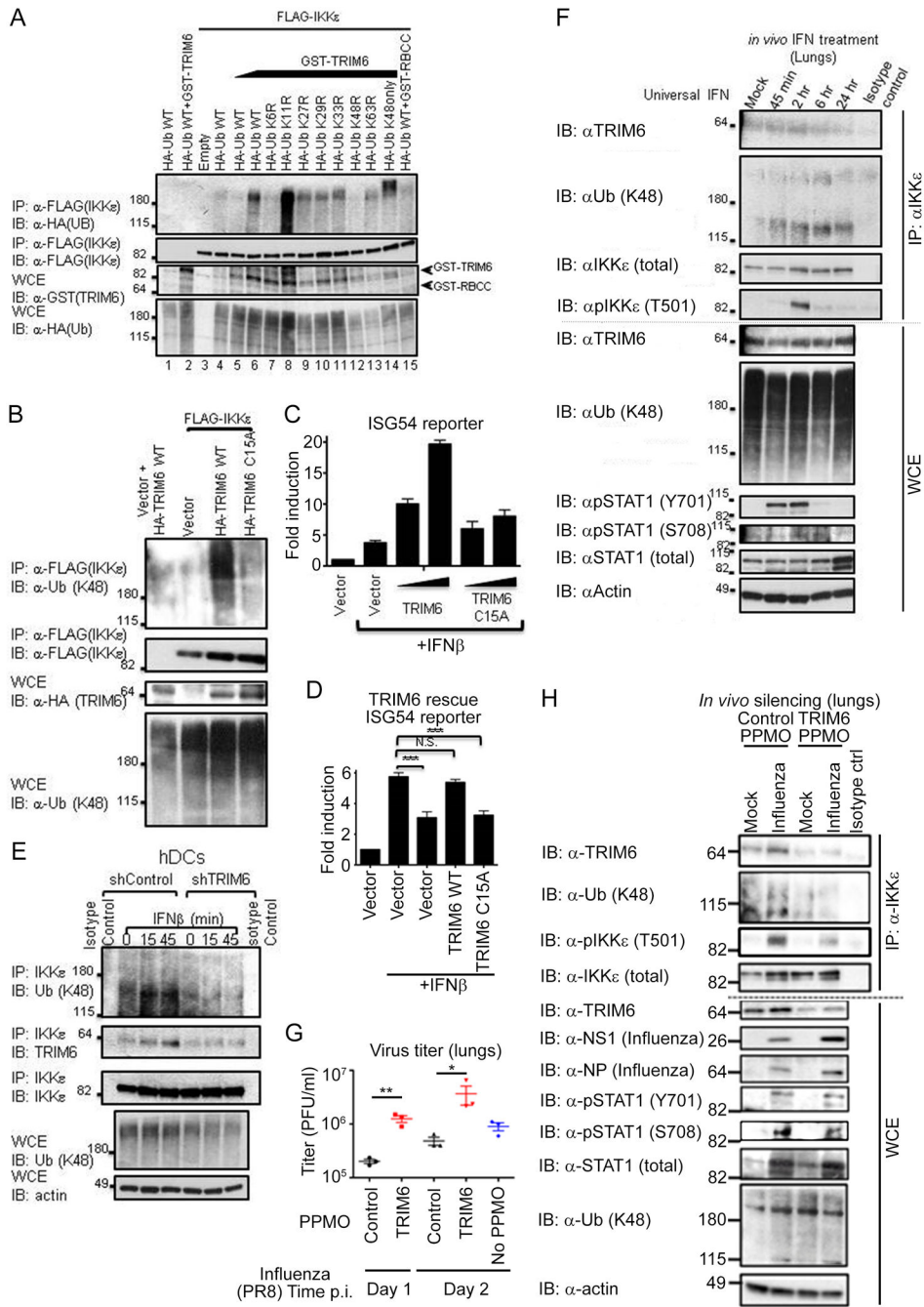
**Figure 3. IKK $\epsilon$  interacts with the C-terminal SPRY domain of TRIM6**

(A) Whole cell extracts (WCE) of HEK-293T cells co-transfected with HA-TRIM6 and either GFP-IRF3, FLAG-IKK $\epsilon$  or TBK-1, were subjected to immunoprecipitation (IP) with anti-HA beads. (B) WCE of hDCs treated with SeV, LPS or IFN $\beta$  were subjected to IP with anti-IKK $\epsilon$  antibody. (C) Localization of HA-TRIM6 and FLAG-IKK $\epsilon$  in HeLa cells determined by confocal microscopy. (D) TRIM6 deletion mutants used for CoIP. (E) GST-pulldown of HEK-293T cells transfected with GST-TRIM deletion mutants and FLAG-IKK $\epsilon$ . See also Figure S3.



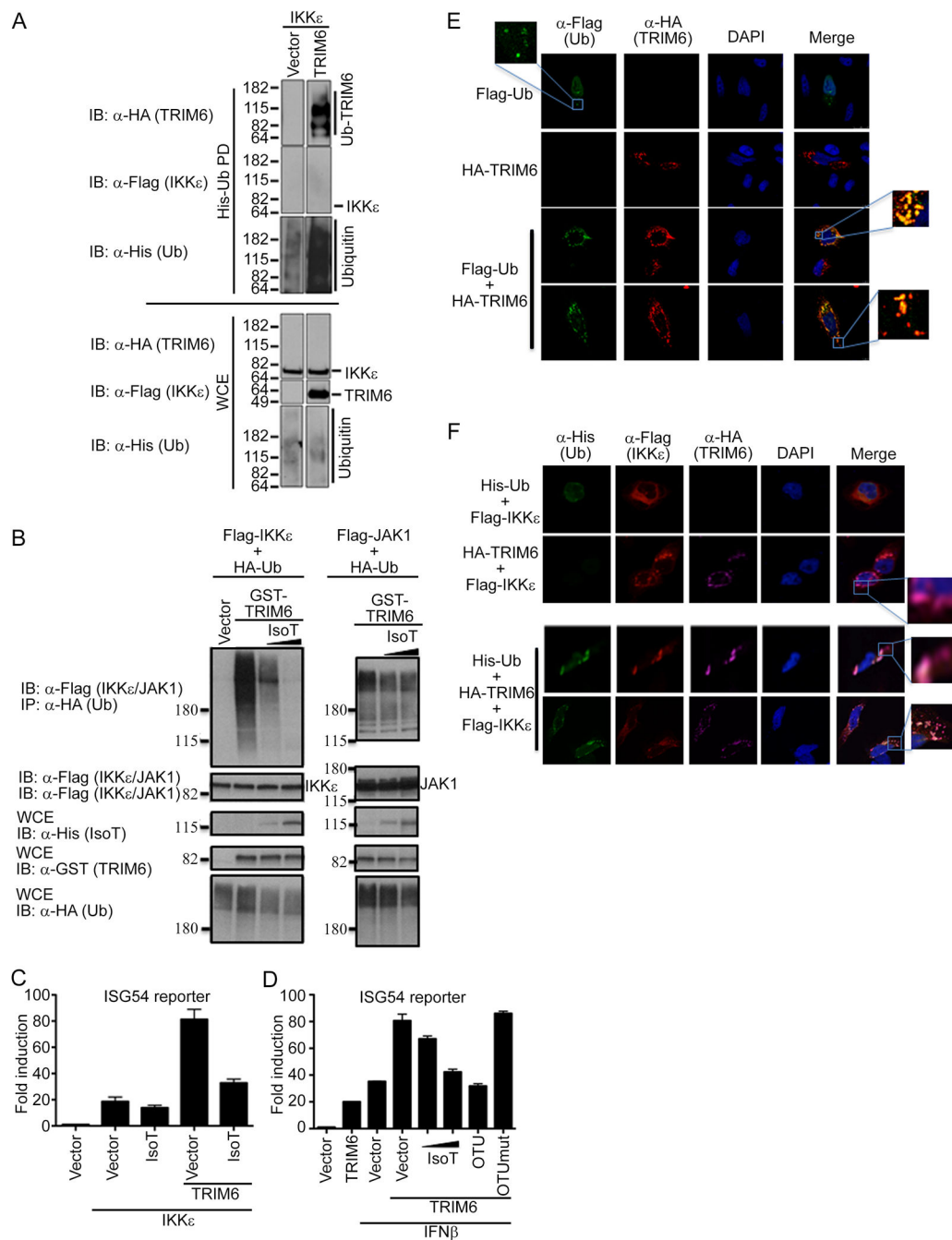


**(F)** EMSA analysis. **(E)** hDCs transduced with lentiviruses expressing TRIM6 shRNAs were stimulated with IFN $\beta$  (100U/ml) and subjected to IB. **(G-H)** A549 cells were transfected with TRIM6 siRNAs for 40 h and subsequently pre-treated with IFN $\beta$  (100U/ml) for 16 h. Cells were then infected with IAV expressing GFP (PR8-GFP) for 16 h. Cells were visualized by fluorescence microscopy **(G)** and quantified by FACS **(H)**. \*p < 0.05; \*\*p < 0.01; \*\*\*p < 0.001, N.S. (not significant). See also Figure S4.



**Figure 5. IKKε interacts with K48-linked poly-ubiquitin chains synthesized by TRIM6**  
**(A)** HEK-293T cells were transfected with FLAG-IKKε together with HA-Ub WT (lanes 1-2, 4-6, 15) or different Ub mutants, and GST-TRIM6 (lane 6-14) or GST-RBCC (TRIM6 lacking SPRY domain: lane 15), or **(B)** FLAG-IKKε with HA-TRIM6 or TRIM6 C15A RING mutant. IP was performed with anti-FLAG beads. **(C)** Luciferase assay of HEK-293T cells transfected with ISG54 reporter together with TRIM6 WT or C15A mutant, and treated with IFNβ (100 U/ml) or **(D)** transfected with TRIM6 siRNA followed by transfection with ISG54 reporter and TRIM6 plasmids and IFNβ treatment (100U/ml) 24 hr later. Depicted is

the mean  $\pm$  SD (n = 3). **(E)** hDCs transduced with lentiviruses expressing TRIM6 shRNAs were stimulated with IFN $\beta$  (100U/ml) and subjected to IKK $\epsilon$  IP. **(F)** BALB/c mice were treated i.n. with 12,000 units of universal IFN. Lungs were collected and IKK $\epsilon$  IP was performed. **(G-H)** Silencing of TRIM6 *in vivo*. BALB/c mice were treated i.n. with TRIM6-targeting or non-targeting PPMO at 24 and 48 hr prior to i.n. infection with 100 pfu IAV PR8. Lungs were collected for virus titer **(G)**, depicted is the mean  $\pm$  SD; n=3, or at 48 hrs pi. for IKK $\epsilon$  IP **(H)**. See also Figure S5.



**Figure 6. IKK $\epsilon$  interacts with unanchored polyubiquitin chains synthesized by TRIM6**  
**(A)** HEK-293T cells, transfected with FLAG-IKK $\epsilon$  plasmid together with empty vector or HA-TRIM6 and His-tagged Ub, were subjected to His-pulldown under denaturing conditions. **(B)** HEK-293T cells were transfected with empty vector or FLAG-IKK $\epsilon$  together with HA-Ub, and GST-TRIM6 in the presence or absence of the isopeptidase T (IsoT) which cleaves only unanchored Ub. At 30 h p.t, IP with anti-FLAG antibody. FLAG-JAK1 was used as a control of covalently bound Ub which IsoT cannot cleave (right side). **(C)** ISG54 reporter assay of HEK-293T cells transfected with IKK $\epsilon$  and TRIM6 in the

presence or absence of IsoT, or **(D)** in the presence or absence of IsoT, OTU domain of the CCHFV or a mutant (C40A and H151A) lacking catalytic activity, followed by IFN $\beta$  treatment. Data shown is representative of 3 independent experiments (mean  $\pm$  SD, n=3). **(E-F)** IKK $\epsilon$  is recruited to “TRIM6 Ub-rich bodies”. **(E)** Confocal microscopy of HeLa cells transfected with HA-TRIM6 and FLAG-Ub, or **(F)** with HA-TRIM6, FLAG-IKK $\epsilon$  and His-Ub. See also Figure S6.





(K38A; **F**) STAT1 and  $\gamma$ 32ATP. The reaction mixture was subjected to SDS-PAGE and visualized by autoradiography. The bands were quantified using ImageJ software. **(G)** Endogenous unanchored poly-Ub chains induce IKK $\epsilon$ -mediated STAT1 phosphorylation. WCE of HEK-293T cells, transfected with FLAG-IKK $\epsilon$  and HA-TRIM6 were subjected to FLAG-IP. Poly-Ub chains bound to IKK $\epsilon$  were isolated by incubation at 75 °C for 5min, followed by treatment with IsoT. These Ub chains were used for IKK $\epsilon$ -mediated STAT1 kinase assay. The bands were quantified using ImageJ software, normalized by the input (Coomassie), and expressed as percentage of control without Ub chains. The quantification data are from 2 independent experiments. See also Figure S7.